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Testing Protocol

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Supplemental Assay Method for the Titration of Feline Panleukopenia Virus in Cell Culture

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Contact: Victor M. Becerra, (515) 663-7468
Marsha J. Hegland, (515) 663-7659

Approvals:

/s/Donna M. Gatewood_____ Date: 09Jun05
Donna M. Gatewood, Section Leader
Virology Section

/s/Byron E. Rippke_____ Date: 13Jun05
Byron E. Rippke, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde_____ Date: 13Jun05
Rebecca L.W. Hyde, Section Leader
Quality Assurance
Center for Veterinary Biologics

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

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Supplemental Assay Method for the Titration of Feline Panleukopenia Virus in Cell Culture

1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying modified-live feline panleukopenia virus (FPV) vaccines for viral content. The method uses the Crandall feline kidney (CRFK) cell line as the test system. Presence or absence of FPV is determined by staining inoculated cell cultures by an indirect fluorescent antibody (IFA) method.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Incubator, 36°± 2°C, high humidity, 5 ± 1% CO_2 (Model 3336, Forma Scientific Inc.)

2.1.2 Incubator, aerobic (Model 2, Precision Scientific)

2.1.3 Water bath

2.1.4 Ultraviolet (UV) light microscope (Model BH2, Olympus America Inc.)

2.1.5 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.1.6 Micropipettor and/or motorized microliter pipette and tips

2.1.7 Glass slides, 8-chamber (Lab-Tek® slides)

2.1.8 Glass staining dish with rack for Lab-Tek slides (glass staining dish)

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 FPV Reference, ICK strain [available from the Center for Veterinary Biologics (CVB)]

2.2.2 CRFK cell culture, free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR) [available from CVB]

2.2.3 FPV Antiserum [available from CVB]

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM with Earles salts without bicarbonate

2.2.4.2 1.1 g sodium bicarbonate ($NaHCO_3$)

2.2.4.3 Q.S. to 1000 ml with deionized water (DW); adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

2.2.4.4 Sterilize through a 0.22-µm filter.

2.2.4.5 Aseptically add 50 µg/ml gentamicin sulfate

2.2.4.6 Store at 2°- 7°C.

2.2.5 Growth Medium

2.2.5.1 920 ml MEM

2.2.5.2 Aseptically add:

1. 70 ml gamma-irradiated fetal bovine serum (FBS)

2. 10 ml L-glutamine (200 mM)

2.2.6 Dulbecco’s phosphate buffered saline (DPBS)

2.2.6.1 8.0 g sodium chloride (NaCl)

2.2.6.2 0.2 g potassium chloride (KCl)

2.2.6.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)

2.2.6.4 0.1 g magnesium chloride, hexahydrate ($MgCl_2 \cdot 6H_2O$)

2.2.6.5 Dissolve reagents in 900 ml DW.

2.2.6.6 Dissolve 1.03 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4) with 10 ml DW, heat to 60°± 2°C until dissolved, then add to **Section 2.2.6.5** with constant mixing.

2.2.6.7 Dissolve 0.1 g calcium chloride, anhydrous ($CaCl_2$) with 10 ml DW and add slowly to **Section 2.2.6.6** to avoid precipitation.

2.2.6.8 Q.S. to 1000 ml with DW, adjust pH to 7.0-7.3 with 2N HCl.

2.2.6.9 Sterilize through a 0.22-µm filter.

2.2.7 Polystyrene tubes, 12 x 75-mm

2.2.8 Appropriate anti-species IgG (H&L) fluorescein isothiocyanate labeled conjugate (Anti-species Conjugate)

2.2.9 100% acetone

2.2.10 Syringe, 1-ml and needle, 20-gauge x 1 1/2-inch

2.2.11 Pipette-aid

2.2.12 Disposable transfer pipette, 3.5-ml

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture as well as in the propagation of animal viruses and the quantitation of virus infectivity by IFA.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of initial titration, set a water bath at 56°± 2°C.

3.2.2 On the day of initial titration, set a water bath at 36°± 2°C.

3.2.3 On the day of the IFA test, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DW.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of CRFK slides

3.3.1.1 Cells are prepared from healthy, confluent CRFK cells, that are maintained by splitting every 3 to 4 days. On the day of test initiation, add 0.4 ml/chamber of approximately $10^{5.2}$ ± $10^{4.9}$ cells/ml diluted in Growth Medium into all chambers of the Lab-Tek® Slides. Prepare sufficient Lab-Tek® Slides to allow 25 chambers for controls and 20 chambers for each Test Vaccine. Incubate at 36°± 2°C in a CO_2 incubator and use within 4 hours. These become the CRFK Slides.

3.3.1.2 Use seeded CRFK Slides within 4 hours.

3.3.2 Preparation of Working FPV Reference

3.3.2.1 On the day of inoculation, rapidly thaw a vial of FPV Reference in a 36°± 2°C water bath.

3.3.2.2 Dispense 1.8 ml of MEM into each of 7, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-7}.

3.3.2.3 Transfer 200 µl of the FPV Reference to the tube labeled 10^{-1}; discard pipette tip. Mix by vortexing.

3.3.2.4 Transfer 200 µl from the 10^{-1} labeled tube to the 10^{-2} tube; discard pipette tip. Mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200 µl from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

3.3.3 Preparation of Working FPV or CPV Antiserum

On the day of the IFA test, dilute FPV or CPV Antiserum in DPBS to the IFA working dilution determined for that specific antiserum.

3.3.4 Preparation of Working Anti-species Conjugate

On the day of the IFA test, dilute Anti-species Conjugate in DPBS to the working dilution according to the manufacturer's recommendations.

3.4 Preparation of the Test Vaccine

3.4.1 The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). On the day of inoculation, rehydrate a vial of the Test Vaccine by transferring 1.0 ml for 1-ml-dose vaccine, 0.5 ml for 1/2-ml-dose vaccine, etc. of the provided diluent into the vial containing the lyophilized Test Vaccine. Use a sterile 1.0-ml syringe and an 18-gauge x 1 1/2-inch needle; mix by vortexing. Incubate for 15 ± 5 minutes at room temperature.

3.4.2 For a monovalent FPV Test Vaccine, mix 200 µl of Test Vaccine with 1.8 ml of MEM in a 12 x 75-mm polystyrene tube for a 10^{-1} dilution. Mix by vortexing.

3.4.3 For a multifraction Test Vaccine containing FPV, heat inactivate the non-FPV fraction(s) in the Test Vaccine in a 56°± 2°C water bath for 60 ± 5 minutes. Dilute according to **Section 3.4.2**.

3.4.4 Dispense 1.8 ml MEM into each of 5, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-6}.

3.4.5 Transfer 200 µl from the Rehydrated Test Vaccine to the tube labeled 10^{-1}; discard pipette tip. Mix by vortexing.

3.4.6 Transfer 200 µl from the tube labeled 10^{-1} to the tube labeled 10^{-2}; discard pipette tip. Mix by vortexing.

3.4.7 Repeat **Section 3.4.6** for each subsequent dilution, transferring 200 µl from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

4. Performance of the test

4.1 Inoculate 5 chambers/dilution of a CRFK Slide with 100 µl/chamber from dilutions 10^{-6} through 10^{-3} of the Test Vaccine. Tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g. 10^{-6} through 10^{-3}).

4.2 Inoculate 5 chambers/dilution of a CRFK Slide with 100 µl/chamber, from dilutions 10^{-6} through 10^{-3} of the Working FPV Reference. Tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g., 10^{-6} through 10^{-3}).

4.3 Five uninoculated chambers serve as negative cell controls.

4.4 Incubate CRFK Slides in a 36°± 2°C CO_2 incubator for 5 days ± 1 day.

4.5 Following incubation, decant the media from the CRFK Slide and remove the plastic wall by twisting them away from the CFRK Slide, leaving the gasket attached to the CRFK Slide.

4.6 Place the CRFK Slides in a slide rack; place the rack in a glass staining dish filled with DPBS. Let stand 15 ± 5 minutes at room temperature.

4.7 Discard the DPBS and fix the CRFK Slides in 100% acetone for 15 ± 5 minutes at room temperature. Remove and allow to air dry.

4.8 Pipette 75 ± 25 µl of the Working FPV Antiserum into each chamber of the CRFK Slides with a transfer pipette. Incubate in the aerobic incubator at 36°± 2°C for 30 ± 5 minutes.

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4.9 Wash per **Section 4.6**. Discard the DPBS.

4.10 Pipette 75 ± 25 µl of the Working Anti-species Conjugate into each chamber of the CRFK Slides; incubate in the aerobic incubator at 36°± 2°C for 30 ± 5 minutes.

4.11 Wash per **Section 4.6**. Discard the DBPS.

4.12 Rinse the CRFK Slides with DW, allow to air dry.

4.13 Read at 100-200X with a UV-light microscope. Examine the cell monolayer for typical apple-green nuclear fluorescence.

4.13.1 Chambers containing one or more cells displaying specific fluorescence for FPV are positive.

4.13.2 Results are recorded as the number of IFA positive chambers versus total number of chambers examined for each dilution of a Test Vaccine and the Working FPV Reference.

4.14 Calculate the FPV endpoints of the Test Vaccine and the Working FPV Reference using the Spearman-Kärber method as commonly modified by Finney. The titer is expressed as $\log_{10}$ 50% fluorescent antibody infective dose ($FAID_{50}$).

Example:

10^{-3} dilution of Test Vaccine = 5/5 chambers FA positive
10^{-4} dilution of Test Vaccine = 4/5 chambers FA positive
10^{-5} dilution of Test Vaccine = 2/5 chambers FA positive
10^{-6} dilution of Test Vaccine = 0/5 chambers FA positive

Spearman-Kärber formula:

Test Vaccine Titer = (X - d/2 + [d • S]) where:

X = $\log_{10}$ of dilution with all chambers IFA positive (3)
d = $\log_{10}$ of dilution factor (1)
S = sum of proportions of chambers IFA positive for all dilutions tested:

$$\frac{5}{5} + \frac{4}{5} + \frac{2}{5} + \frac{0}{5} = \frac{11}{5} = 2.2$$

Test Vaccine titer = (3 - 1/2) + (1 • 2.2) = 4.7

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Adjust the titer to the Test Vaccine dose as follows:

A. divide the **Test Vaccine Dose** by the **Inoculation Dose**

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this FPV test vaccine, the recommended dose is 1 ml)

Inoculation Dose = amount of diluted Test Vaccine added to each chamber of the Test Slide (for this FPV test vaccine, the inoculation dose is 0.1 ml)

$$\frac{\text{1 ml dose}}{\text{0.1 ml inoculum}} = 10$$

B. calculate $\log_{10}$ of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

Log of 10 = 1.0

Test Vaccine titer = 4.7 + 1.0 = 5.7

Therefore the titer of the **FPV Test Vaccine** is $10^{5.7}$ $FAID_{50}$/ml.

5. Interpretation of the test results

5.1 Validity requirements:

5.1.1 The calculated titer of the Working FPV Reference must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The uninoculated cell controls must not exhibit any cytopathic effect, specific FPV fluorescence, or cloudy media that would indicate contamination.

5.1.3 The lowest inoculated dilution of the FPV Reference Control must produce a FA positive reaction in 100% of the chambers (5/5). If an endpoint is not reached, (1 or more chambers are FA positive at the highest dilution), the titer is expressed as "greater than or equal to" the calculated titer. If an endpoint is critical to testing, the highest (most dilute) must exhibit no positive FA reaction (0/5).

5.1.4 If the validity requirements are not met, then the assay is considered a **NO TEST** and can be retested without prejudice.

5.1.5 If the validity requirements are met and the titer of the Test Vaccine is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Vaccine is considered **SATISFACTORY**.

5.1.6 If the validity requirements are met but the titer of the Test Vaccine is less than the required minimum titer contained in the APHIS filed Outline of Production for the product under test, the Test Vaccine is retested in accordance with 9 CFR 113.8.

6. Report of test results

Results are reported as the $FAID_{50}$ per dose of Test Vaccine.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.304, U.S. Government Printing Office, 2004.

7.2 Cottral G.E., (Ed.), 1978. *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY. pg. 731.

7.3 Finney, D.J. 1978. *Statistical methods in biological assay.* Griffin, London. 3rd edition, pp. 394-401.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.2** Removed self-refilling syringe from the list of Reagents/supplies needed.

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- **2.2.4.2** The amount of sodium bicarbonate ($NaHCO_3$) has been changed from 2.2 g to 1.1 g.

- **2.2.4.5** Penicillin and streptomycin have been deleted.

- **4.14** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.

- **5.1.3** Clarification of the endpoints for a valid assay has been added.

- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.

- "Test Serial" has been changed to "Test Vaccine" throughout the document.

- "Tissue culture infective dose ($TCID_{50}$)" has been changed to "fluorescent antibody infective dose ($FAID_{50}$)" throughout the document.

- The footnotes have been deleted with any pertinent references now noted next to the individual items.